THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal ☐ Second Renewal ☒ Date: July 30, 1973

1. Principal Investigator (give title and degrees).
Una S. Ryan, Ph.D. (formerly Una Smith), Senior Scientist,
Papanicolaou Cancer Research Institute and
Assistant Professor of Medicine, University of Miami School of Medicine

2. Institution & address:
Papanicolaou Cancer Research Institute
1155 N.W. 14th Street
Miami, Florida 33136

Mailing Address: P.O. Box 6188
Miami, Florida 33123

3. Department(s) where research will be done or collaboration provided:
Cardiopulmonary Unit, Sieron Building
1425 N.W. 10th Avenue
Miami, Florida 33136

1003541911

4. Short title of study:
THE ROLE OF ENDOTHELIAL AND EPITHELIAL CELLS IN NON-VENTILATORY FUNCTIONS OF THE LUNGS

5. Proposed renewal date: January 1, 1974

6. How results to date have changed earlier specific research aims:
In general, our studies have produced few unexpected results in terms of the pulmonary processing of angiotensin I, bradykinin and the adenine nucleotides. In consequence, our specific aims have changed to allow a progressively finer focus on the precise subcellular localizations of the relevant enzymes. As indicated in our previous application, we have broadened the scope of the research program to include studies of type II alveolar epithelial cells, their lamellar inclusions and the modulations produced by hormones and drugs. We propose to continue these studies. However, in the previous application, we outlined experiments to examine the subcellular sites of pulmonary enzymes capable of degrading prostaglandins. To date, it has not been possible to perform the indicated experiments as we deliberately chose to pursue what we regarded as more promising leads in our studies of endothelial cells.

7. How results to date have changed earlier working hypothesis:
As in our previous applications, we intend to continue testing the hypothesis that pulmonary endothelial cells are actively engaged in the metabolism of circulating angiotensin I and bradykinin. The major focus for the coming year will be to determine the precise subcellular localization of angiotensin I converting enzyme by cytoimmunologic techniques using monospecific antibodies to hog lung converting enzyme.

The second major research goal is to examine the fine structure of type II alveolar cells and to examine the modulations of these cells and their lamellar inclusions by hormones and drugs such as nicotine.

8. Any additional facilities now required? Describe briefly:

We do not anticipate the need of additional major items of capital equipment.

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

Peter C. Moller, Ph.D., has joined our staff as a co-investigator. Dr. Moller replaces Dr. Doris Chang, who returned to Taiwan. Dr. Moller has experience in both electron microscopy and cell culture. He will assist with the electron microscopy required for the proposed program and will also have the primary responsibility for scaling up cultures of pulmonary endothelial cells.

10. Append outline of experimental protocol for ensuing year.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

Smith, D.S., Smith, U. and Ryan, J.W.: Freeze-etched lamellar body membranes of the rat lung great alveolar cell. Tissue & Cell, 4:457, 1972.

Smith, U., Ryan, J.W. and Smith, D.S.: Freeze-etch studies of the plasma membrane of pulmonary endothelial cells. J. Cell Biol., 56:492, 1973.

Ryan, J.W. and Smith, U.: The metabolism of angiotensin I by endothelial cells. In Vol. 20 Protides of the Biological Fluids (ed. H. Peeters), Pergamon Press, Oxford, England, 1973, pp. 379-384.

Smith, U., Smith, D.S. and Ryan, J.W.: Tubular myelin assembly in type II alveolar cells: Freeze-fracture studies. Anat. Rec., 176:125, 1973.

Smith, U., Smith, D.S., Winkler, H. and Ryan, J.W.: Exocytosis in adrenal medulla demonstrated by freeze-etching. Science, 179:79, 1973.

(Continued on Page 2a)

12. Summary progress report (append in standard form as separate document, unless recently submitted).

Publications Continued:

Smith, U. and Ryan, J.W.: Electron microscopy of endothelial cells collected on cellulose acetate paper. Tissue & Cell, 5:333, 1973.

Smith, U. and Ryan, J.W.: Electron microscopy of endothelial and epithelial components of the lungs: Correlations of structure and function. Fed. Proc., in press.

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)		% time	Amount
*Una Ryan, Ph.D.	Principal Investigator	25%	
**J.W. Ryan, M.D., D.Phil.	Co-Investigator	10%	
**Peter C. Moller, Ph.D.	Co-Investigator	50%	REDACTED
Fringe Benefits			
Technical			
Sharon Monticone	Lab Tech II	100%	REDACTED
Erica Clements	Lab Tech	50%	
Fringe Benefits			
			REDACTED
		Sub-Total for A	

B. Consumable supplies (by major categories)	
Animals: rats $400, rabbits $200, Golden hamsters $100	700
Photographic supplies	1,000
Chemicals and glassware, general	800
Fixatives, embedding and staining materials	700
Cell culture media, culture chambers and compress gas tank	1,200
Sub-Total for B	4,400

C. Other expenses (itemize)	
Travel	
Una Ryan	500
J.W. Ryan	500
Peter C. Moller	500
Shipping charges	150
Reference Books	75
Regrinding diamond knives for microtomy	350
Sub-Total for C	2,075
Running Total of A + B + C	REDACTED

D. Permanent equipment (itemize)	
Durst glassless negative carrier	80
Photographic easel	60
Sub-Total for D	140
E. Indirect costs (15% of A+B+C)	4,596
Total requ st	REDACTED

BUDGET JUSTIFICATION

*Una Ryan (formerly Una Smith, please see letter to Dr. Hockett, dated July 30, 1973). The major portion of Dr. Una Ryan's salary is paid by the American Heart Association through her tenure of an Established Investigatorship. The salary requested here is calculated as 25% of the allowed supplement.

**James W. Ryan and Peter C. Moller. Salary funds are requested in proportion to per cent times to be spent on this research project. Dr. P.C. Moller replaced Dr. Doris Chang, who has returned to Taiwan. Dr. Moller's biographical sketch is attached.

Fringe benefits, including social security, health insurance, life insurance and unemployment, are calculated as 10% of salaries.

14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Endothelium: Structure and Functions (salary only)	American Heart Association (72 160)	15,000 (02) 16,000 (03) 17,000 (04) 18,000 (05)	July 1, 1973 to June 30, 1976
*Studies on Normal Lung Cell Separation, Culture and Morphology	National Heart and Lung Institute NO1-HL-3-3015-LD	164,900 (of which $97,000 is designated for the purchase of a Philips 301 electron microscope)	July 1, 1973 to Sept. 30, 1974

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
It is not yet known whether renewal funds will be available for the NHLI contract program (NO1-HL-3-3015-LD). If such funds become available, renewal will be requested.			

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*The work solicited by the contract of the National Heart and Lung Institute does not overlap in approach, concept or timing with the research program described in the present application. However, the EM 301 electron microscope will be available to the studies proposed here for funding by the Council of Tobacco Research.

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Una S. Ryan

Signature Una S. Ryan Date 7/30/73

Telephone 305 (Area Code) 373-5903 (Number) (Extension)

Checks payable to

Papanicolaou Cancer Research Institute

Mailing address for checks:

P.O. Box 6188

Miami, Florida 33123

Responsible officer of institution

Typed Name Dr. Julius Schultz

Title Director and President

Signature [signature] Date 31 July 73

Telephone 305 371-5572 34

(Area Code) (Number) (Extension)

Proposed Research

Fine structural localization of the angiotensin I converting enzyme

As discussed in the summary progress report and in previous progress reports, all existing evidence indicates that angiotensin I and bradykinin are metabolized by enzymes on or close to the luminal surface of pulmonary endothelial cells. However, it has not proved possible to test the concept by any single existing technique. Previously, we showed that the plasma membrane fraction of whole lung homogenate does in fact convert angiotensin I to angiotensin II and degrades bradykinin to characteristic lower homologs (Ryan and Smith, 1971; Ryan et al., 1972). Although these data support the hypothesis, we do not know what proportion of the plasma membrane is derived from endothelial cells and what proportion comes from other cell-types. Therefore, we subsequently began efforts to isolate endothelial cells to obtain direct evidence of their metabolic capabilities (Ryan and Smith, 1973).

Results with isolated endothelial cells of the mainstem pulmonary artery are described below (section 12) in greater detail. Our results show that pure monolayers of endothelial cells can in fact convert angiotensin I to angiotensin II. While the ability of a pure line of endothelial cells to metabolize angiotensin I adds strength to our hypothesis on the subcellular site of the relevant enzymes, we believe that two further studies are required to round out definitive tests.

Firstly, in our studies of pulmonary endothelial cells isolated on cellulose acetate paper, we found means of carrying monolayers of cells in primary culture. Thus the possibility exists of obtaining endothelial cells in sufficient quantities, through scaled-up cell culture, to allow harvesting of pure plasma membrane fractions from a known cell line. Given sufficient starting material, we propose to use the method developed previously in this laboratory (Ryan and Smith, 1971) to isolate plasma membrane and to test the reactivities of the preparation with angiotensin I and bradykinin.

As a second approach, we have agreed to a collaborative study with Dr. Frederic Dorer, Cleveland Veterans Hospital, to raise specific antibodies to the converting enzyme of hog lung. Dorer and colleagues (1972) have succeeded in purifying the enzyme to homogeneity and we have begun the requisite immunizations. Although we project booster immunizations, we have already obtained an antibody reactive with angiotensin converting enzyme as demonstrated by the Ouchterlony technique. Further analysis is required, but evidence on hand indicates that the antibody is monospecific. The antibodies will be used in an attempt to establish the subcellular site of converting enzyme by cytoimmunologic techniques. For the purposes of electron microscopy, antibody will be labelled with ferritin in one series of experiments and with horse radish peroxidase in a second series. Ferritin is itself electron-dense after reaction with OsO_4. The peroxidase, on reaction with diaminobenzidine, will yield an electron-dense product suitable for high magnification studies.

Judging from similar studies conducted by others for other purposes (Moriarty and Halmi, 1972), we believe it likely that the proposed cytoimmunologic

investigations can show a precise localization of angiotensin I converting enzyme. Resolution of the order of 100Å should be achievable using either ferritin or peroxidase.

Type II alveolar cells

It is clear from our previous studies that further understanding of the genesis, maturation and release of lamellar bodies of type II alveolar cells will require major improvements in the preservation of phospholipid components during preparation of tissues for electron microscopy. We believe that these improvements will be of particular importance in understanding the effects of hormones and drugs, such as nicotine, on lamellar bodies and other organelles of type II cells.

Technical developments. One of the most striking features brought out by replicas of freeze-etched material is the extreme regularity of the leaflets in the lamellar bodies. Using several variations of standard dehydration procedures necessary for embedding, we, as others, have been unable to show the regular organization in thin sections. Indeed in most published pictures, lamellar bodies have looked like vacuoles containing some lamellae at their periphery. However, all available cytochemical and biochemical evidence indicates that the lamellar bodies and surfactant are predominantly lipid in nature. Sorokin (1967) suggested that the lamellar bodies are imperfectly preserved by conventional osmium-aldehyde fixation followed by processing in lipid solvents. An important phospholipid component of surfactant, dipalmitoyl lecithin, would clearly be vulnerable to conventional specimen preparation (Dermer, 1969).

Aqueous embedding media, avoiding alcoholic dehydration, should greatly enhance the preservation of lamellar bodies, however published protocols give inconsistent results and poor sectioning properties. A method suggested recently by Prof. R. Barrnett (personal communication) incorporates a rational approach which we shall pursue first. In Barrnett's method, tissue is fixed in glutaraldehyde (usually we use 2.5% glutaraldehyde in cacodylate buffer, pH 7.4). The polymerization reaction mixture is prepared, on ice, as follows: A solution of glutaraldehyde (50-75%) is mixed vigorously with pure carbohydrazide (m.p. 153°C, Eastman-Kodak or Polysciences) to give a final concentration of carbohydrazide of 3.3M. The preparation can then be frozen at -20°C or can be used immediately for embedding. When used for embedding, the glutaraldehyde-carbohydrazide solution is diluted serially to give solutions in water of 25%, 50%, 70%, 85%, 95% and 100%. Fixed tissues are moved through these serial dilutions until they have been exposed to the 100% solution. Then a few drops of the undiluted glutaraldehyde-carbohydrazide solutions are applied to dental wax, the tissue is added and polymerization begins at room temperature. After 8 hours at 37°C, the block can be applied to a chuck and processed from that point in the usual fashion.

In addition to preserving the regular organization of the leaflets of intracellular lamellar bodies, the aqueous embedding method should also preserve the organization of extracellular lamellar bodies and the airspace reticulum and surface film.

Embedding techniques which preserve lipids as they exist in vivo should have great impact on the remainder of the research proposal. Firstly, it will allow a firm baseline for the search, using thin sections, needed to confirm substructures revealed by freeze-etching. Secondly, since shadowing materials of unknown thickness are used in freeze-etch techniques, the most reliable measurements of substructural components and their spacing should be provided by examination of thin sections. Thirdly, freeze-fracture techniques use more tissue in a more random manner (one accepts the fracture wherever it occurs) than do standard techniques. Therefore, studies of well-preserved thin sections give more control—as will be required for serial sections, cytochemistry and immunocytochemistry.

We believe that cytochemistry will be a very valuable ancillary procedure for approaching a variety of problems concerning the origins, maturation and fate of lamellate bodies. For example, during its intracellular life, the lamellar body is known to contain a spectrum of enzymes including acid phosphatase, (Hatasa and Nakamura, 1965; Goldfischer et al., 1968; Meban, 1972) alkaline phosphatase (Sorokin, 1967) and esterases (peptidase enzymes?) capable of hydrolyzing p-nitrophenylthiol esters (Vatter, et al., 1968). On the other hand, there is little or no information on what happens to these enzymes when the lamellar inclusion is expelled into the airspace. Possibly all enzymatic activity is lost. If this is so, the point should be established. However, if the enzymes survive in the airspace, it would be important to demonstrate their survival as there may be functions of expelled enzymes in terms of remodeling the surface lining or the reticulum or even in the processing of organic inhalants such as those which may be contained in tobacco smoke. Having developed methods of preserving the airspace lining, we propose to use standard methods for acid and alkaline phosphatase (Gomori, 1952) and the methods of Vatter et al., for esterase enzymes. The studies will be coordinated with investigations of enzymic activities of lung lavage fluid rendered acellular by differential centrifugation. Cell organelles and reticular substances will be purified by sucrose density gradient ultracentrifugation (Steim et al., 1969). Lipids of the supernatant will be examined by thin layer chromatography (Steim et al., 1969) and phospholipids will be measured in terms of chloroform-extractable phosphorus (Brown et al., 1964).

We further believe that selective staining techniques may bring information to bear on the mechanisms by which expelled lamellar bodies unfold. Drawing on studies of the expulsion of the contents of mucocysts of Tetrahymena (Satir et al., 1972), once fusion of the cyst with the plasma membrane has occurred, there is an immediate release of the highly-concentrated mucoid contents. Satir postulates that biological energy is not required. The entry of water followed by hydration of the mucoid would greatly expand the material, forcing it out of the stoma and into the surrounding media.

Possibly, the analogy applies to lamellar bodies, which are known to contain sialomucin (Luke and Spicer, 1965). We propose, using ruthenium red (a selective stain for acidic polysaccharides, Luft, 1964, 1965), to examine lamellar bodies within and without the giant alveolar cell for the disposition of acidic polysaccharides. Although the results would not, by themselves, be conclusive a disposition of acidic polysaccharides between the lipid leaflets should, on hydration in the airspace, cause the leaflets to spread. Furthermore, a hydrated

reticulum may have the effect of "floating" the surface lipid. On this point, ruthenium red has been used in previous studies of airspace contents (Brooks, 1969), but without the benefit of an embedding medium which preserves lipid membrane. Ruthenium red both stains and precipitates acidic polysaccharides, and therefore there should be no major losses during aqueous embedding.

Freeze-etch studies. The major effort in further freeze-etch studies is to extend our previous findings of lamellar organization and substructure of the leaflets in a manner to allow precise measurements. It should be emphasized that while freeze-etching can produce an elegant demonstration of structure and substructure, one cannot control exactly where fractures will occur. The only feasible approach to obtaining a full range of fracture planes is to make large numbers of replicas using large numbers of different tissue blocks prepared by several different means (high glycerol content, low glycerol, etc.). Clearly, this approach requires considerable time. As we described under Technical developments (above), the further development of aqueous embedding media will also take a great deal of time. We therefore plan to start making replicas immediately so that, as suitable thin sections become available, coordinated studies may proceed apace. This need not involve a hazard in deterioration of material as the metallic freeze-etch replicas are relatively durable.

We plan to find means of examining the unfractured membrane surfaces of giant alveolar cells and of lamellar bodies. For these experiments, glycerol, the usual cryoprotectant, will be omitted or reduced to low concentrations to permit lowering of the water table by sublimation. Etching will be continued for up to 10 minutes. Visualization of membrane surfaces as well as their fracture faces may allow a further understanding of the disposition of the globular particles presumed to be intramembranous (Branton, 1971), and their possible relationships to the release of lamellar inclusions. In other systems organization of intramembranous particles appears to be intimately related to membrane fusion and exocytosis (Satir et al., 1971; Smith et al., 1973).

As mentioned above, replicas are of uncertain thickness and therefore one cannot be certain of the validity of measurements derived from what is, in fact, a coating of the original structure. However, it now appears feasible using a film thickness monitor which has just become available (Balzers, Lichtenstein) to make replicas of reproducible thickness, thus making it possible to compare one replica with the next even though absolute measurements may be in doubt. Comparative measurements can then be made between the periodicity of extracellular tubular myelin and the substructural array of particles on lamellar body leaflets. Measurements of the spacing of tubular myelin in thin sections show a 400Å-500Å lattice which corresponds with the freeze-etch repeating pattern. Furthermore, it is important to establish the interlamellar distance, which on the basis of our present measurements, appears to be characteristic for this organelle and may be a function of chemical composition.

Relationship of lamellar bodies to other organelles of the giant alveolar cell. The close relationship between lamellar bodies and mitochondria has been illustrated by our pilot study. However, other laboratories have suggested that lamellar bodies arise in association with multivesicular bodies (Vatter et al., 1968), in the "cytoplasmic vesicle" (Hatasa and Nakamura, 1965) and in mitochondria

(Bargmann and Knoop, 1956). In terms of origin and in terms of the progressive maturation of lamellar bodies up until the time of their release from the cell, it is important to determine the spatial and possible structural relationships to other organelles. Using the complexity of the substructural array seen in freeze-etching as an index of maturity, it may be possible to determine the stages in the intracellular processing of surfactant material and to document contributions from other organelles. The sequence of events in the synthesis, through subsequent release of zymogen (from rough ER through Golgi apparatus to exocytosis) from the exocrine pancreas (Palade and Siekevitz, 1956) could be taken as an example of such a life history.

The possible origins of lamellar bodies from mitochondria has been debated for many years. Although we see no clear conclusions, some reviewers (Scarpelli, 1968) have pointed out that mitochondria and lamellar bodies have different histochemical reactivities and have taken this as conclusive evidence that lamellar bodies could not arise from mitochondria. In the light of more recent evidence (Werb and Cohn, 1972) on the formation of phagolysosomes, structures derived from plasma membrane and lysosomes, it is evident that histochemical reactions may be quite different at late stages of evolution from those of contributing structures.

We believe it to be unlikely that the instances we have found of mitochondria and lamellar bodies enveloped by a single membrane are fortuitous. Myelin figures, which in thin section have some resemblance to lamellar bodies, are known to be formed by mitochondria under certain adverse conditions (see for e.g., Tuchweber et al., 1972). However, the envelopment of lamellar bodies and mitochondria by a single membrane can be interpreted in a variety of different ways. The broadest interpretation is that communications occur between these organelles, and the communication may or may not be the consequence of one organelle evolving to the next. Taking previous cytochemical studies into account with our findings, we regard it as important to make a systematic search for communications of lamellar bodies with other organelles, most prominently multivesicular bodies (Vatter et al., 1968) and lysosomes (Hatasa and Nakamura, 1965). In this manner we intend to provide further information on the assembly of materials ultimately expelled into the airspace.

Having located transitional forms in the search just described, we will use selective cytochemical techniques [succinic dehydrogenase for mitochondria, (Ogawa and Barrnett, 1965) acid phosphatase for lysosomes, (Essner and Novikoff, 1961) etc.] to determine which enzymes lamellar bodies lose or gain during their intracellular life cycle. These studies will be coordinated with the investigation described above for examining enzymes of the airspace reticulum.

Effects of hormones. Studies by Redding et al. (1972) and Olsen (1972) indicate that thyroid and some neurohumoral agents can profoundly influence the synthesis and secretion of lamellar bodies. If thyroid hormone does in fact stimulate synthesis and assembly, cells under the influence of thyroid hormone should show more transitional forms, a point that would greatly facilitate our studies on the maturation of lamellar bodies. Similarly, examination of fetal lung may provide the same opportunity. In addition, the effects of isoproterenol on secretion will facilitate examination of mechanisms of delivery of the lamellar body to the cell membrane. There is the further point, of far greater importance, that the effects of these hormones could possibly have therapeutic implications.

Our first studies will use essentially the same protocol as that of Redding et al. (1972). Rats will be made hyperthyroid by daily subcutaneous injections of 1 mg L-thyroxine per kilogram for 6 days. Thyroid status will be monitored by assay of serum-protein-bound iodine and by following body weight. We will incorporate additional studies using triiodothyronine. In addition, studies on the absence of hormone will be accomplished using rats 6-8 weeks after thyroidectomy. We will coordinate the morphologic studies with studies of lung lavage fluid as described by Redding et al., 1972. Studies of fine structure of the giant alveolar cells and their lamellar inclusions will be conducted as described above (freeze-etch replicas and thin sections), while monitoring Clara cells and macrophages.

Synthetic glucocorticoids may well accelerate maturation of giant alveolar cells (Olsen, 1972). In addition, it is known that the lungs take up as much aldosterone as do the kidneys (Sulya et al., 1963). Therefore we propose to conduct a similar series of investigations incorporating controls, adrenalectomized animals and adrenalectomized animals with specific replacement therapy (cortisol, corticosterone, desoxycorticosterone or aldosterone).

The effects of inhaled nicotine may well be of interest in terms of the fine structure of giant alveolar cells and in terms of the fate of lamellar bodies. As described previously, Olsen (1972) has shown that pilocarpine causes acute depletion of lamellar bodies of giant alveolar cells while increasing the phospholipid content of lung lavage fluid. These effects are prevented or partially inhibited by atropine, a point suggesting that the pilocarpine effect is muscarinic (in terms of the classification of acetycholine-like effects). Possible nicotinic effects have not been tested, but could be of importance in terms of direct cellular effects or parasympathetic synaptic effects.

Chlorphentermine may present another opportunity. If, as existing data indicate, chlorphentermine stimulates the rate of synthesis of lamellar bodies, chronic administration of the drug to experimental animals should enhance our ability to identify the major stages of genesis, maturation and secretion of these inclusions.

Although it is highly-speculative at the moment, it is possible that chlorphentermine interferes with cholesterol synthesis. Dr. Lüllmann-Rauch (personal communication) has found that triparanol, a well-studied inhibitor of cholesterol synthesis, also induces the accumulation of airspace "foam" cells and of free lamellar forms. This point can be considered with the findings by Werb and Cohn (1972) which indicate that membrane synthesis requires exogenous cholesterol. Possibly, when cholesterol is not available, other membrane components, such as phospholipid, accumulate in excess.

Summary Progress Report - March 16, 1972 - July 31, 1973.

The primary objectives of our research are

1) to examine the relationships of fine structure of pulmonary endothelial cells to the selective processing of circulating hormones, and

2) to examine the effects of hormones and drugs on the fine structure of type II alveolar cells and to study possible participation of type II alveolar cells in the processing of substances of the prostaglandin type.

Structure and function of pulmonary endothelial cells

As discussed in earlier progress reports, all available evidence supports the concept that bradykinin and angiotensin I, like the adenine nucleotides, are metabolized by enzymes on or close to the surface of pulmonary endothelial cells. Our hypothesis is based on the findings that angiotensin I and bradykinin disappear during a single circulation through the lungs (Ryan et al., 1970 and 1971). Disappearance is owing to enzymic degradation and not to tissue uptake nor transfer to extravascular spaces. Specific metabolites are recovered in nearly quantitative yields in the pulmonary venous effluent. Blood enzymes play little or no role as angiotensin I and bradykinin are degraded no less rapidly during circulation through lungs freed of blood.

Using bradykinin or angiotensin I labelled intrinsically with ^{14}C or ^{3}H, the apparent volumes of distribution and mean transit times of radioactivity do not exceed those of blue dextran (MW>2,000,000), a compound unlikely to leave the intravascular space (Ryan et al., 1972). Furthermore, we have found that angiotensin I and bradykinin are degraded to characteristic products by the plasma membrane-caveolae intracellulares fraction of whole lung homogenates.

However, endothelial cells of the pulmonary capillaries are extremely thin (as little as 0.01 μ) and because of this thinness and because of the variety of cell types within the lungs, it could be argued that rapid uptake or transfer of angiotensin, followed by rapid release of metabolites might be difficult to detect in our experiments. Therefore we decided to study the metabolism of angiotensin I by pure monolayers of endothelium collected from mainstem pulmonary artery.

We obtained Häutchen preparations of endothelium by applying strips of cellulose acetate paper to the luminal surface of the pulmonary artery. We have shown that cells obtained by this method are viable in short-term tissue culture. The coherence of the endothelium can be demonstrated by staining the paper and attached cells with methylene blue and cleaning in xylene. Electron microscopy presented a problem since the cellulose acetate paper is soluble in many of the preparative solvents. Nevertheless, by developing an agar sandwich technique (Smith and Ryan, 1973) we were able to examine the cells in the electron microscope and further to demonstrate that the endothelium occurred as a pure monolayer and was not contaminated by other cell types or extracellular material. The endothelial cells show a preferential attachment to the cellulose acetate paper and we were unable to separate them. However, we were able to set up the

entire preparation (cellulose acetate support and endothelial monolayer) as a vital chromatographic system such that angiotensin I was applied to the top of the support and angiotensin II was collected in the eluate. These data were presented at a colloquium "Protides of the Biological Fluids" in Brugge, Belgium (Ryan and Smith, 1973).

These results, in combination with our earlier findings that the plasma membrane/caveolae fraction of whole lung homogenates metabolized bradykinin, angiotensin I and the adenine nucleotides as does whole lung (Ryan and Smith, 1971), and that 5'-nucleotidase activity is localized in endothelial caveolae, all point to the endothelial plasma membrane as the site of enzymic activity. We have summarized this work in a review to the American Physiological Society, September, 1972, to be published: Smith and Ryan, Fed. Proc., 1973.

As discussed in the proposal of research submitted last year, we undertook studies on the fine structure of pulmonary endothelial cells. We found, as has been described for other cells studied by freeze-fracture techniques, that intramembranous particles (70-100Å in diameter) are distributed randomly on both fracture faces of undifferentiated plasma membrane. However, the particles organize in rings and plaques at the base of caveolae and appear to adhere predominantly to the outer leaflet. The specific organization of particles in association with caveolae confirms the presence of a skeletal supporting rim to caveolae, a point previously suggested by our studies of thin sections (Smith and Ryan, 1972). We also identified particles in association with the outer leaflet of the caveola membrane itself which may represent the globular substructures which we reported in thin-sectioned material and which we regard as likely candidates for enzyme clusters. Our freeze-fracture studies raise the question as to whether there is a relationship between the organization of intramembranous particles and the structure of endothelial caveolae. Whether the particles can organize as rings or plaques in response to stimuli and thus be related to the mechanism of initiation of pinocytosis is an intriguing possibility.

The organization and preferential adherence of intramembranous particles of pulmonary endothelial cells may well be germane to our studies of the localization of enzymes which metabolize circulating vasoactive substances. As will be discussed more fully in section 10 (Proposed research), Dr. F. Dorer and colleagues at the Cleveland Veterans Administration Hospital have isolated converting enzyme from hog lungs. The molecular weight of this enzyme is approximately 300,000. Assuming a globular configuration, an enzyme of this size could readily be accomodated in a particle 70-100Å in diameter. Dr. Dorer has made the enzyme available to us and descriptions of its use in our proposed experiments appear in greater detail in section 10.

Type II alveolar cells and surfactant

Part of the commitment of our current research is to study the normal structure and function of type II alveolar cells and their relationships to the surface active lining of the lung. The long-term aim of these studies is to provide a data base for understanding the effects of hormones and drugs (such as nicotine) on type II cells and for understanding the role of these cells in the processing of surfactant and possibly inhalants.

We have undertaken a study in which we coordinated examination of thin sections with that of replicas of freeze-fractured material (D.S. Smith et al., 1972; U. Smith et al., 1973). Studies of thin sections confirmed previous reports that lamellar bodies are released into the alveolar space where they appear to disassemble to yield tubular myelin, a component of surfactant (see micrograph). Freeze-etch replicas reveal the intracellular lamellar bodies as highly structured, in which lamellae are arranged concentrically or in parallel rows. Fractured lamellae bear particles approximately 100Å in diameter which are remarkable for their organization in an array of parallel rows and ribs exactly resembling the periodicity (400-450Å) of the lattice of tubular myelin as it occurs in the airspace. We have suggested that the ribs exposed within the lamellar body may represent tubular myelin elements in the course of assembly (D.S. Smith et al., 1972; Smith and Ryan, 1973). Myelin and synthetic lamellar phase phospholipids invariably fracture to yield smooth surfaces (Deamer et al., 1970; Branton, 1971). The fracture faces of the lamellar body resemble other freeze-fractured biological membranes in containing intramembranous particles. Following the correlation made by Branton (1971), the presence of abundant particles may reflect corresponding physiological activity. Some fracture planes reveal incomplete rows of particles and suggest an entirely new set of morphological criteria for evaluating the maturation of lamellar bodies in addition to supporting the view that a component of surfactant is synthesized within the characteristic inclusions of the type II alveolar cell, prior to its exocrine release into the airspace.

Related Studies

Although the metabolism of bradykinin in the pulmonary circulation results in a complete inactivation, the metabolism of angiotensin I is in fact primarily an activation reaction. The major polypeptide metabolite of angiotensin I is angiotensin II, the most potent hypertensive agent known. In addition to its effects on the tone of peripheral vascular beds, angiotensin II is considered to be a highly selective secretogogue capable of stimulating the release of aldosterone from the adrenal cortex and catecholamines from the adrenal medulla (Laragh et al., 1960; Feldberg and Lewis, 1964). Thus it appears likely that the lungs, by processing angiotensin I, can influence specific activities of the adrenal gland.

Over the past year, we carried out a collaborative study with Dr. Hans Winkler, University of Innsbruck, on the effects of angiotensin II on the adrenal medulla and on the mechanism of release of catecholamines from medullary chromaffin cells. The effects of angiotensin II on the medulla are dramatic. Feldberg and Lewis (1964) have estimated that one molecule of angiotensin II may cause the release of 5,000 molecules of epinephrine. Over the past ten years, data has accrued indicating that epinephrine is contained in membrane-limited granules and is released from chromaffin cells by a process called exocytosis. The concept of exocytosis was based largely on morphological descriptions and the biochemical evidence that the contents of chromaffin granules are released to the extracellular space in the absence of a concomitant release of cytoplasmic substances or of membrane components (Smith and Winkler, 1972).

Using freeze-fracture techniques, we have demonstrated pockets indicating points of attachment and fusion of chromaffin granules with the plasma membrane.

Of importance to our original goals, we further found that the frequency of the points of fusion is increased in glands perfused with angiotensin II and is vastly decreased when calcium is omitted from the perfusion system (Rubin, 1970). So far as we are aware, our studies are the first to demonstrate a discrete ultrastructural effect of angiotensin II.

Significance

Although gas exchange between blood and air is undoubtedly the fundamental function of the alveolar capillary unit, there is a growing body of direct and indirect evidence which indicates that the endothelial cells and type II alveolar cells carry out specialized metabolic functions of possible importance not only to the performance of the lungs themselves but also to activities of distant organs and glands. Our previous studies of these "non-ventilatory" functions suggest that it is feasible to relate the functions to fine structure by using advanced techniques of tissue preparation and high magnification electron microscopy.

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Figure Caption

Electron micrograph showing lamellar bodies giving the appearance of unravelling to yield tubular myelin after expulsion into the air space.

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CURRICULUM VITAE

Name: Peter Christian Moller

Birth date and place: **REDACTED**

Marital Status:

REDACTED

Education and Degrees:

University of Houston, Houston, Texas -- 1965 — B.Sc. Biology
Rice University, Houston, Texas -- 1971 — Ph.D. Cell Biology

Thesis Advisor: Dr. Charles W. Philpott

Thesis Title: The Pharyngeal Circulatory System of Amphioxus: Fine Structure and Cytochemical Studies of the Vascular System in a Cephalochordate.

Research and/or Professional Experience:

1965-1967	Chief Technician, Cell Biology and EM Laboratory, Department of Biology, Rice University, Houston, Texas.
1967	Instructor in Introduction to Biology, Rice University, Houston, Texas.
1968	Instructor in Botany Laboratory, Rice University, Houston, Texas.
1969	Instructor in Cellular Physiology Laboratory, Rice University, Houston, Texas.
1971 (summer)	Postdoctoral Fellowship, Department of Biology, Rice University, Houston, Texas (With Dr. J.W. Campbell).
9/71-5/73	Research Fellow, Division of Biological and Medical Sciences, Brown University, Providence, Rhode Island (With Dr. Richard A. Ellis).
6/73-present	Research Scientist, Papanicolaou Cancer Research Institute, Miami, Florida.

Military Service:

1957-1959 United States Coast Guard

Academic and Professional Honors:

Fellow-Trainee, U.S.P.H.S., Rice University, 1967-1971.
Postdoctoral Fellowship, U.S.P.H.S., Rice University, 1971 (summer).
Postdoctoral Fellowship, U.S.P.H.S., Brown University, 1971-1973.

[redacted]

Membership in Scientific Societies:

REDACTED REDACTED

Presentations:

October 1968 - Presentation to the Texas Society for Electron Microscopy.
January 1969 - Demonstration to the Texas Society for Electron Microscopy (by invitation).
April 1970 - Presentation to the American Association of Anatomists, Chicago, Illinois.
May 1971 - Presentation to the Division of Biological and Medical Sciences, Brown University (by invitation).
May 1971 - Presentation to Dr. S.S. Spicer's group, Department of Pathology, Medical University of South Carolina.
December 1971 - Presentation to the Department of Zoology, University of Rhode Island (by invitation).

Abstracts:

November 1971 - The circulatory system of Amphioxus, Abstract, The Eleventh Annual Meeting of the American Society for Cell Biology, New Orleans, Louisiana.

Publications:

1) Moller, P.C. and Philpott, C.W.: The circulatory system of Amphioxus (Branchiostoma floridae) I. Morphology of the major vessels of the pharyngeal area. J. Morph., 139:389-406, 1973.

2) Moller, P.C. and Philpott, C.W.: The circulatory system of Amphioxus II. Uptake of exogenous proteins by endothelial cells. Z. Zellforsch., in press.

3) Moller, P.C. and Ellis, R.A.: The excretory system of Amphioxus. Submitted to Am. J. Anat.